

**Amendments to the Claims:**

This listing of claims will replace all prior versions, and listings, of claims in the application:

**Listing of Claims:**

- 1-34. (cancelled)
35. (currently amended) A modified pneumolysin polypeptide comprising one or more amino acid substitutions, wherein the modification of the in a pneumolysin polypeptide comprises substituting at least one amino acid of having a sequence of SEQ ID NO:3, and wherein said substitution is wherein single amino acid substitutions occur at a position selected from the group consisting of positions 61, 148, and 195, or wherein substitutions of multiple more than one amino acids occur at positions selected from the group consisting of 17, 18, 33, 41, 45, 46, 61, 63, 66, 83, 101, 102, 128, 148, 189, 195, 239, 243, 255, and 257, and wherein when said modified pneumolysin polypeptide possesses only one substitution, said substitution is selected from the group consisting of positions 61, 148, and 195, and wherein said modified pneumolysin polypeptide having at least one amino acid substitution is soluble, elicits antibodies which are cross-reactive with wild-type pneumolysin, and has attenuated hemolytic activity.
36. (previously presented) The polypeptide according to claim 35, wherein the hemolytic activity is less than 25% compared to wild-type pneumolysin.
37. (previously presented) The polypeptide according to claim 36, wherein the hemolytic activity is less than 1% compared to wild-type pneumolysin.
- 38-41. (cancelled)
42. (previously presented) The polypeptide according to claim 35, wherein the polypeptide is selected from the group consisting of pNVJ1, pNVJ20, pNVJ22, pNVJ45, pNVJ56, pNV103, pNV207, pNV111, and pNV211.
43. (previously presented) Modified pneumolysin polypeptide pNVJ1.
44. (previously presented) Modified pneumolysin polypeptide pNVJ20.

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We claim:

1 A modified pneumolysin polypeptide having attenuated hemolytic activity wherein said modified pneumolysin polypeptide is obtained by:

- Ans B1*
- 5 a) randomly mutating a nucleic acid molecule encoding for wild-type pneumolysin to produce mutated nucleic acid molecules encoding modified pneumolysin polypeptides and expressing the mutated nucleic acid molecules in host cells;
- 10 b) assaying the modified polypeptide expressed by the host cells for hemolytic activity;
- c) identifying the modified pneumolysin polypeptides having substantially similar molecular weight as native wild-type pneumolysin and which are refoldable.

*Ans B2*

20 2. A modified properly-refolded pneumolysin polypeptide having attenuated hemolytic activity comprising an amino acid sequence of type 14 pneumolysin wherein at least one amino acid in the region comprising amino acid residues 1 to 257 is substituted and wherein at least one of said amino acid substitutions results in attenuation of the hemolytic activity of the modified pneumolysin polypeptide.

- 25 3. The modified pneumolysin polypeptide of claim 2, wherein the hemolytic activity is less than 25% compared to wild-type pneumolysin.
4. A modified pneumolysin polypeptide according to claim 3, comprising at least one amino acid substitution in the amino acid sequence of Formula I at residue positions 61, 148, or 195 or the combination of substitutions at residue positions 33, 46, 83, 239 and 257, said Formula I comprising
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(Formula I)

Met Ala Asn Lys Ala Val Asn Asp Phe Ile Leu Ala  
1 5 10  
5 Met Asn Tyr Asp Lys Lys Leu Leu Thr His Gln  
15 20  
Gly Glu Ser Ile Glu Asn Arg Phe Ile Lys Glu Gly  
25 30 35  
Asn Gln Leu Pro Asp Glu Phe Val Val Ile Glu Arg  
10 40 45  
Lys Lys Arg Ser Leu Ser Thr Asn Thr Ser Asp Ile  
50 55 60  
Ser Val Thr Ala Thr Asn Asp Ser Arg Leu Tyr Pro  
65 70  
15 Gly Ala Leu Leu Val Val Asp Glu Thr Leu Leu Glu  
75 80  
Asn Asn Pro Thr Leu Leu Ala Val Asp Arg Ala Pro  
85 90 95  
20 Met Thr Tyr Ser Ile Asp Leu Pro Gly Leu Ala Ser  
100 105  
Ser Asp Ser Phe Leu Gln Val Glu Asp Pro Ser Asn  
110 115 120  
Ser Ser Val Arg Gly Ala Val Asn Asp Leu Leu Ala  
125 130  
25 Lys Trp His Gln Asp Tyr Gly Gln Val Asn Asn Val  
135 140  
Pro Ala Arg Met Gln/Tyr Glu Lys Ile Thr Ala His  
145 150 155  
30 Ser Met Glu Gln Leu Lys Val Lys Phe Gly Ser Asp  
160 165  
Phe Glu Lys Thr Gly Asn Ser Leu Asp Ile Asp Phe  
170 175 180  
Asn Ser Val His Ser Gly Glu Lys Gln Ile Gln Ile  
185 190  
35 Val Asn Phe Lys Gln Ile Tyr Tyr Thr Val Ser Val  
195 200  
Asp Ala Val Lys Asn Pro Gly Asp Val Phe Gln Asp  
205 210 215  
40 Thr Val Thr Val Glu Asp Leu Lys Gln Arg Gly Ile  
220 225  
Ser Ala Glu Arg Pro Leu Val Tyr Ile Ser Ser Val  
230 235 240  
Ala Tyr Gly Arg Gln Val Tyr Leu Lys Leu Glu Thr  
245 250  
45 Thr Ser Lys Ser Asp Glu Val Glu Ala Ala Phe Glu  
255 260  
Ala Leu Ile Lys Gly Val Lys Val Ala Pro Gln Thr  
265 270 275

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Glu Trp Lys Gln Ile Leu Asp Asn Thr Glu Val Lys  
280 285  
Ala Val Ile Leu Gly Gly Asp Pro Ser Ser Gly Ala  
290 295 300  
5 Arg Val Val Thr Gly Lys Val Asp Met Val Glu Asp  
305 310  
Leu Ile Gln Glu Gly Ser Arg Phe Thr Ala Asp His  
315 320  
Pro Gly Leu Pro Ile Ser Tyr Thr Thr Ser Phe Leu  
10 325 330 335  
Arg Asp Asn Val Val Ala Thr Phe Gln Asn Ser Thr  
340 345  
Asp Tyr Val Glu Thr Lys Val Thr Ala Tyr Arg Asn  
350 355 360  
15 Gly Asp Leu Leu Leu Asp His Ser Gly Ala Tyr Val  
365 370  
Ala Gln Tyr Tyr Ile Thr Trp Asn Glu Leu Ser Tyr  
375 380  
Asp His Gln Gly Lys Glu Val Leu Thr Pro Lys Ala  
20 385 390 395  
Trp Asp Arg Asn Gly Gln Asp Leu Thr Ala His Phe  
400 405  
Thr Thr Ser Ile Pro Leu Lys Gly Asn Val Arg Asn  
410 415 420  
25 Leu Ser Val Lys Ile Arg Glu Cys Thr Gly Leu Ala  
425 430  
Trp Glu Trp Trp Arg Thr Val Tyr Glu Lys Thr Asp  
435 440  
Leu Pro Leu Val Arg Lys Arg Thr Ile Ser Ile Trp  
30 445 450 455  
Gly Thr Thr Leu Tyr Pro Gln Val Glu Asp Lys Val  
460 465  
Glu Asn Asp (50 20 10:3) no Period.

35 5. The modified pneumolysin according to claim 4,  
wherein a single amino acid substitution is made and  
the substituted amino acid is selected from the group  
consisting of proline or hydroxyproline for position  
B 61, lysine, arginine or histidine for position 148 and  
40 leucine, glycine, alanine, isoleucine or valine for  
position 195.

6. The modified pneumolysin according to claim 3,  
wherein the substituted amino acids are selected from

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the group consisting of serine, threonine,  
asparagine, glutamine, tyrosine or ~~cysteate~~ for  
positions 33, 46 and 83; lysine, arginine or histidine  
for position 239 and leucine, glycine, alanine,  
isoleucine or valine for position 255.

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7. Modified pneumolysin polypeptide pNVJ1.
8. Modified pneumolysin polypeptide pNVJ20
9. Modified pneumolysin polypeptide pNVJ22.
10. Modified pneumolysin polypeptide pNVJ45.
- 10 11. Modified pneumolysin polypeptide pNVJ56.
12. Modified pneumolysin polypeptide pNV103.
- B* 13. Modified pneumolysin polypeptide pNV207.
14. Modified pneumolysin polypeptide pNV111.
15. Modified pneumolysin polypeptide pNV211.
- 15 16. A recombinant nucleic acid molecule encoding a  
modified type 14 pneumolysin polypeptide wherein at  
least one amino acid in the region comprising amino  
acid residues 1 to 257 is substituted and wherein at  
least one of said amino acid substitutions results in  
attenuation of the hemolytic activity of the modified  
pneumolysin polypeptide.
- 20 17. The recombinant nucleic acid molecule according to  
claim 16 comprising the following pneumolysin nucleic  
acid sequence:

25 ATGGCAAATA AAGCAGTAAA TGACTTTATA CTAGCTATGA  
ATTACGATAA AAAGAAACTC TTGACCCATC AGGGAGAAAG

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T-134→C, A-305→G, A-566→G and T-583→G;

T-583→G;

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T-583→A;

T-443→A;

and

10 T-181→C.

18. The recombinant nucleic acid molecule of claim 16 as contained in a vector such as a plasmid, cosmid, bacteriophage or yeast artificial chromosome.

15 19. A microorganism comprising the nucleic acid molecule of claim 16.

20. The microorganism according to claim 19, wherein the microorganism is selected from the group consisting of bacteria, yeast, mammalian or insect cells.

21. The microorganism according to claim 20, wherein the microorganism is *E. coli*.

B 22. The modified pneumolysin polypeptide of claim 1, <sup>Claim 2</sup> 9 wherein the polypeptide is conjugated to a polysaccharide which elicits antibodies cross-reactive with a bacterial polysaccharide.

25 23. The modified pneumolysin conjugate of claim 22, wherein the polysaccharide is from a bacteria selected from the group consisting of a Haemophilus influenzae type b; meningococcal group A, B or C; group B streptococcus types Ia, Ib, II, III, V or

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- VIII and pneumococcal.
24. A vaccine comprising at least one pneumolysin polypeptide of claim 1 and a pharmaceutically acceptable carrier.
- 5 25. The vaccine according to claim 24, wherein the polypeptide is conjugated to a polysaccharide which elicits antibodies cross-reactive with a bacterial polysaccharide.
- 10 26. The vaccine according to claim 25, wherein the polysaccharide is derived from a bacteria selected from the group consisting of Haemophilus influenzae type b; meningococcus group A, B, or C; group A streptococcus or group B streptococcus serotypes Ia, Ib, II, III, V, or VIII; or one or more of serotypes 1-23 of *S. pneumoniae*.
- 15 27. A method for killing bacteria comprising contacting said bacteria with antibodies to an immunogenic molecule comprising the modified pneumolysin according to claim 1 in the presence of complement.
- 20 28. The method according to claim 27, wherein the immunogenic molecule is a polysaccharide-polypeptide conjugate wherein the polysaccharide is a bacterial capsular polysaccharide.
- 25 29. A method for immunization of mammals comprising administering the vaccine of claim 24 to said mammals.
30. A method for obtaining modified pneumolysin polypeptides having reduced hemolytic activity and